Evidence of AT_1R-AT_2R-RXFP1 Functional Crosstalk in Myofibroblasts and its Impact on the Therapeutic Targeting of Renal and Cardiac Fibrosis

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Running title: AT_1R-AT_2R-RXFP1 functional crosstalk

SUPPLEMENTAL MATERIAL

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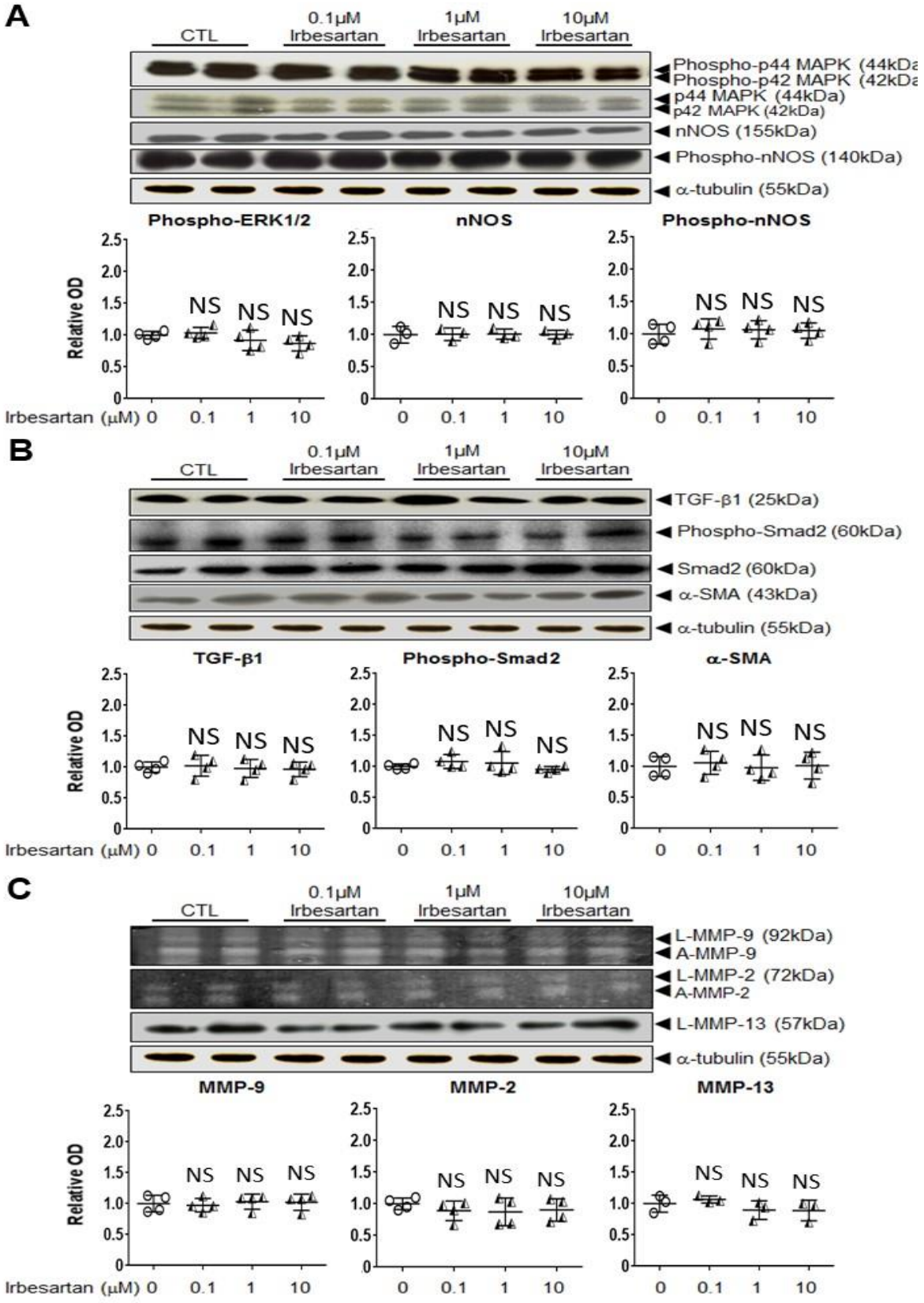
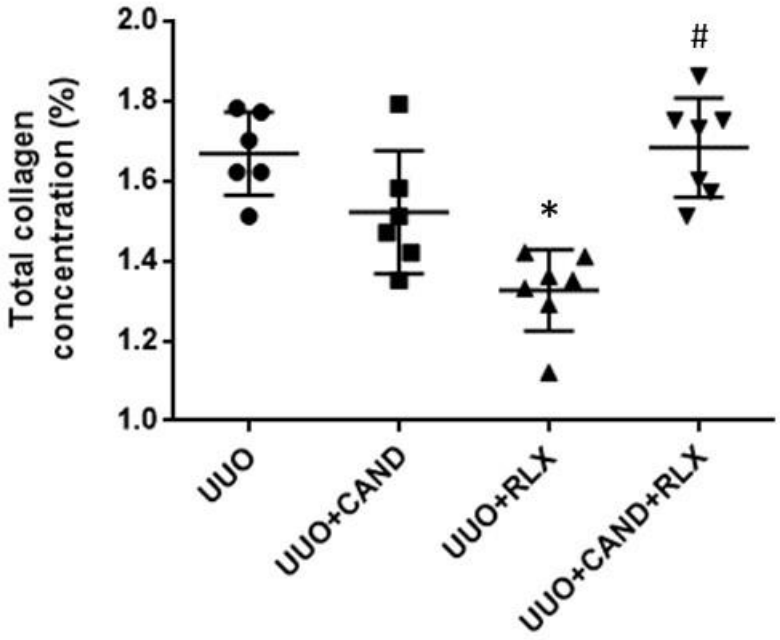
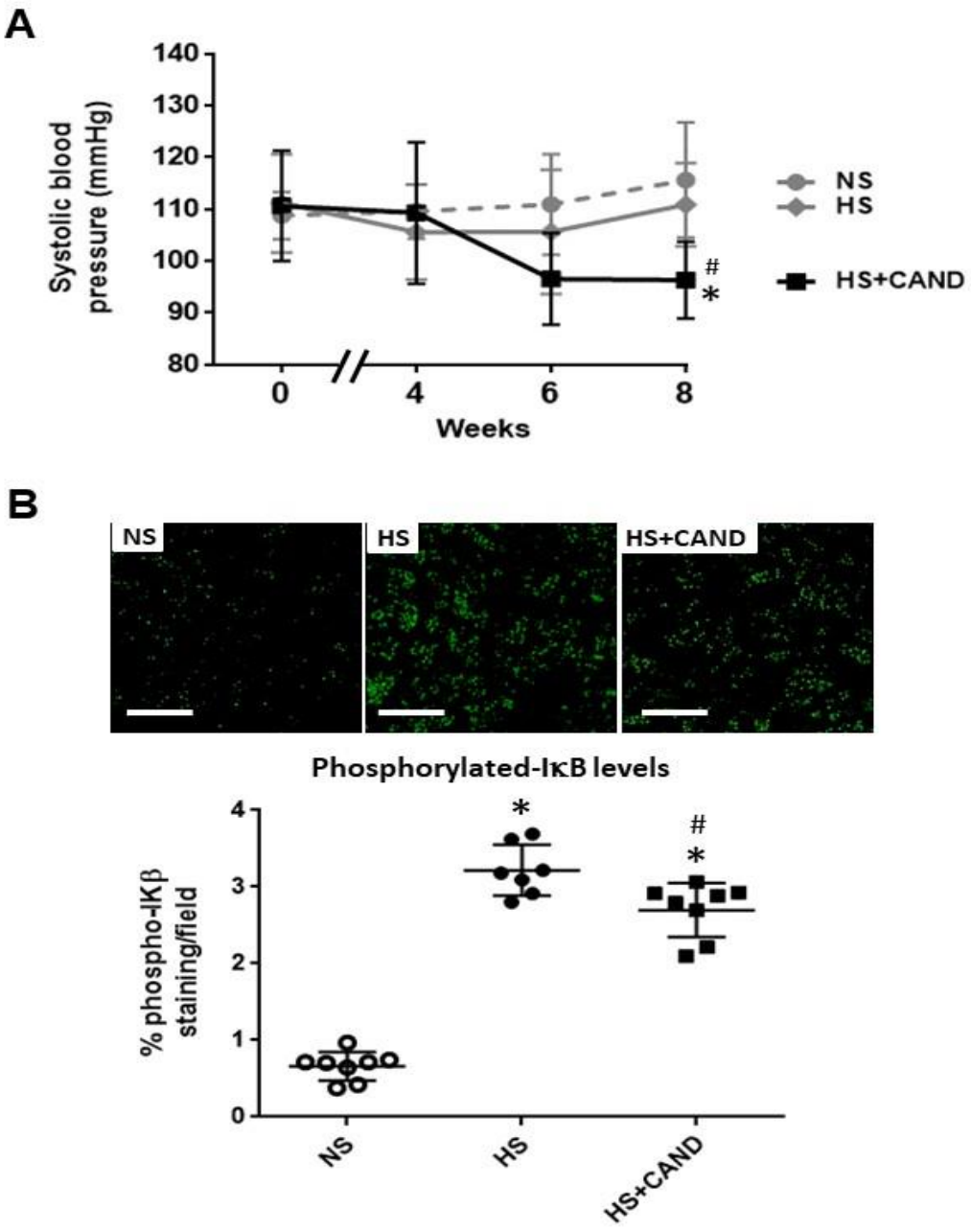


Supplemental Figure 1. Irbesartan does not affect the signal transduction end-points associated with the anti-fibrotic actions of RLX, in the absence of RLX. (A) Representative Western blots of renal phosphorylated (phospho)-p44 and p42 MAPK (phospho-ERK1/2), total p44 and p42

MAPK (ERK1/2), nNOS, phospho-nNOS, and α-tubulin; (B) TGF-β1, phospho-Smad2, total Smad2, α-SMA and α-tubulin; and (C) representative gelatin zymographs of latent (L) and active (A) MMP-9 and MMP-2 levels and representative Western blots of L-MMP-13 and α-tubulin expression from untreated (control) rat renal myofibroblasts and cells treated with irbesartan (0.1, 1, 10nM) after 72 hours in culture. The total (A) p44 and p42 MAPK (ERK1/2), (B) unphosphorylated Smad2, and (A-C) α-tubulin blots were included to demonstrate the quality and equivalent loading of protein samples. Also shown are the relative mean ± SEM optical density (OD) levels of (A) phospho-ERK1/2 (corrected for total ERK1/2 levels), nNOS, and phospho-nNOS (both corrected for α-tubulin levels); (B) TGF-β1, α-SMA (both corrected for α-tubulin levels), and phospho-Smad2 (corrected for total Smad2 levels); and (C) MMP-9, MMP-2, and MMP-13 (corrected for α-tubulin levels) from each of the groups studied, as determined by densitometry scanning (from n=3-4 separate experiments conducted in duplicate), to that of the untreated group, which was expressed as 1 in each case. NS denotes not significantly different compared to values from the untreated control group.



Supplemental Figure 2. Candesartan alone did not reduce renal fibrosis post-UUO at the time-point studied. Total renal collagen concentration (as determined from hydroxyproline analysis) was evaluated 5 days post-UUO in sub-groups of untreated mice vs those treated with candesartan (2mg/kg/day; via drinking water) alone, RLX (0.5mg/kg/day; via osmotic mini-pumps) or both combined; with all treatments administered from 2 days prior to UUO until 5 days post-UUO (from n=6-7 animals per treatment group). Candesartan alone only induced a trend towards preventing UUO-induced renal collagen concentration, potentially due it its slow-acting effects in this model[54], but was able to significantly abrogate the collagen-inhibitory effects of RLX. *$p<0.05$ vs UUO alone; $^{\#}p<0.05$ vs UUO+RLX group.



Supplemental Figure 3. Candesartan alone reduced systolic blood pressure and renal inflammation post-HS-induced nephropathy. (A) Candesartan (2mg/kg/day; via drinking water) administration to high salt (HS; 5% NaCl)-fed mice, from weeks 5-8 of the 8 week model, significantly lowered systolic blood pressure (after 4 weeks of administration; at week 8). (B) Candesartan also significantly lowered the HS-induced increase in phosphorylated-IκB immunostaining (which was used as a surrogate marker of NF-κB activity) after 4 weeks of administration (at week 8) (n=6-8 animals per treatment group); indicative of its therapeutic efficacy in this model. Scale bar = 50μm.*$p<0.05$ vs normal salt (NS)-fed group; $^{\#}p<0.05$ vs HS-fed group.